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Prepared by K. Ritalahti, Q. Wu, and F. Löffler, Environmental Engineering, Georgia Institute of Technology

BENCH SCALE EVALUATION OF ANAEROBIC MICROBIAL DEGRADATION OF HEXACHLOROCYCLOPENTADIENE AND OCTACHLOROCYCLOPENTENE

The feasibility of bioremediation approaches for the removal of hexachlorocyclopentadiene (C-56) and octachlorocyclopentene (C-58) at the former Occidental Chemical Corporation (OCC) facility in Montague, Michigan, was evaluated.

The purpose of the bench-scale testing was to explore if indigenous microbes are capable of degrading the target contaminants C-56 and C-58, and if bioaugmention is a feasible approach to accelerate the rate of contaminant removal. This information is relevant to decide on the most promising and cost-efficient remediation strategy for contaminant containment and removal.

This report includes six individual reports:

Report 1: Microbial Analysis of Sediment Materials and Groundwater
Report 2: Microbial Analysis of Microcosms After 206 Days of Incubation
Report 3: Contaminant Analysis of Sediment Materials and Groundwater
Report 4: Evaluation of Anaerobic Microbial Degradation of Hexachlorocyclopentadiene (C-56) and Octachlorocyclopentene (C-58) in Sediment and Groundwater
Report 5: Abiotic investigations to determine the fate and transport of C-56 and C-58
Report 6: Further Abiotic Investigations.

Report 1: Microbial Analysis of Sediment Materials And Groundwater

- Performed by Kirsti Ritalahti, Ph.D.

OBJECTIVE

Explore the presence of known reductively dechlorinating bacterial populations using 16S rRNA gene-based tools in sediment and groundwater samples collected at the former Occidental Chemical Corporation (OCC) facility in Montague, Michigan. The samples were collected in January 2004 and shipped on blue ice in a cooler to Georgia Tech.

Table 1. Sediment samples provided January of 2004

Sample ID (GaTech)	Analyzed	Matrix
ETC 1-3, 08/Jan/04	24/Mar/04	solids 97-04 (clean), 3 x 1g
ETM 1-3, 08/Jan/04	24/Mar/04	solids 25-1A (mild), 3 x 1g
ETD 1-3, 08/Jan/04	24/Mar/04	solids 02-03B (DNAPL), 3 x 1g

Table 2. Groundwater samples provided January of 2004

Sample ID (GaTech)	Analyzed	Matrix
ETGWC, 08/Jan/04	24/Mar/04	groundwater 97-04 (clean), 2L
ETGWM, 08/Jan/04	24/Mar/04	groundwater 25-1A (mild), 2L
ETGWD, 08/Jan/04	24/Mar/04	groundwater 02-03B (DNAPL), 2L

METHODS

DNA isolation. Triplicate 1 g sediment samples were aseptically transferred to MoBio Soil Extraction vials, and DNA was extracted as per manufacturer recommendation (MoBio Laboratories Inc., Carlsbad, CA). Two liters of groundwater were filtered through a polyether sulfone membrane filter (0.2 µm pore size), and DNA extractions were performed using the MoBio Water DNA kit (MoBio). The DNA preparations from both the solids and groundwater extractions were concentrated to total volumes of 50 µl, and DNA was quantified by spectrophotometry at a wavelength of 260 nm.

Detecting known dechlorinating bacterial groups. Two qualitative poymerase chain reaction (PCR)-based approaches were used. The PCR technique is essentially a primer extension reaction to create exponentially increasing quantities of a specific nucleic acid sequence in vitro. The direct PCR approach uses community DNA as template. This method has a detection limit of roughly 1,000 16S rRNA gene copies per µl of PCR reaction mix. For more sensitive detection of dechlorinating bacteria present in smaller numbers, the nested PCR approach can be used. In this case, general (universal) bacterial primers are used to amplify community 16S rRNA genes in an initial PCR reaction, prior to a second round of amplification with dechlorinator 16S rRNA gene-targeted primers. Only a few copies of the 16S rRNA gene(s) of interest need be present in the original sample to be detected with the nested PCR approach.

Community DNA was extracted from all samples, and used as template in PCR with primer pairs that specifically target the 16S rRNA genes of each of five dechlorinating bacterial groups (*Dehalococcoides* spp., *Desulfuromonas* spp., *Dehalobacter* spp., *Desulfitobacterium* spp. and *Desulfomonile* spp.) (1, 3, 4, 9, 13). Positive controls included genomic DNA of *Dehalococcoides* sp. strain FL2, *Desulfuromonas michiganensis* strain BB1 (DSM 15941), *Dehalobacter restrictus* (DSM 9455), *Desulfitobacterium* sp. Viet1, and *Desulfomonile tiedjei* (DSM 6799) for the *Dehalococcoides-*, *Desulfuromonas-*, *Dehalobacter-*, *Desulfitobacterium-* and *Desulfomonile-* targeted primers, respectively. Gel electrophoresis was used to visualize amplicons corresponding to the targeted groups of dechlorinating bacteria (amplicon size given in parenthesis): *Dehalococcoides* (620 bp), *Desulfuromonas* (815 bp), *Dehalobacter* (828 bp), *Desulfitobacterium* (624 bp), and *Desulfomonile* (423 bp).

For increased sensitivity in detecting dechlorinating bacteria, the nested PCR approach was used. First, an initial amplification of the communities' 16S rRNA genes was performed using universal bacterial primers 8F and 1541R and 20-30 ng of community DNA as template as described (15). Following amplification, 2 µl of PCR product was analyzed by agarose gel electrophoresis to verify that 16S rRNA genes could be amplified from the community DNA. Then, 1.25 µl of 1:2 and 1:50 dilutions of the 16S rRNA gene amplicons were used as templates in PCR with the specific primer pairs in a second round of PCR (i. e., nested PCR).

Quantifying Dehalococcoides spp. Real-Time (RTm) PCR was used to estimate the number of *Dehalococcoides* cells present in the samples (3). RTm PCR uses a linear oligonucleotide probe that contains a reporter dye at its 5' end and a quencher dye at its 3' end. The reporter signal is blocked by the quencher dye as long as the probe remains intact. When the probe is cleaved, the dye and the quencher dissociate and the reporter dye fluoresces when stimulated with the appropriate wavelength of light. During amplification,

the probe anneals to the middle portion of the target sequence and is then cleaved by the 5' exonuclease activity of Taq polymerase as it extends the sequence from an upstream primer. Thus, a probe is cleaved each time a primer is extended and the fluorescent signal increases in proportion to the amount of PCR product generated. The spectrofluorimetric thermal cycler (ABI Prism 7700 Sequence Detection System, Applied Biosystems) records the emitted fluorescence intensities of each reaction at regular intervals, then compares these values to a standard curve to determine the initial target copy number. The number of gene targets per ml of groundwater is determined using a standard curve generated with known concentrations of the target gene (i.e., the *Dehalococcoides* 16S rRNA gene). The *Dehalococcoides* 16S rRNA gene-targeted primers and probe set quantifies total *Dehalococcoides* individuals at a particular site, assuming one 16S rRNA gene copy per *Dehalococcoides* genome (3).

Detecting and quantifying indicator genes. The 16S rRNA gene-based approach does not always distinguish between *Dehalococcoides* populations with distinct dechlorinating activities. Hence, analysis of the *tceA* gene implicated in trichloroethene-to-vinyl chloride reductive dechlorination, and the *bvcA* gene implicated in vinyl chloride-to-ethene reductive dechlorination were included in the analysis. Each gene exists in a single copy on the *Dehalococcoides* genome, and the quantification of the target genes translates directly into cell numbers.

RESULTS

The samples listed in Tables 1 and 2 were used for PCR analyses. When 2 L of groundwater were concentrated, there were obvious color differences in the membrane filters between the three samples. The midrange contaminant groundwater had a bright yellow hew, while the "clean" site had the lightest coloration.

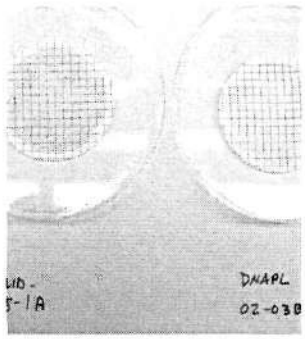


Table 3. DNA extracted from groundwater and sediment samples.

Source	Absorbance 260 nm	Absorbance 280 nm	Ratio 260/280	DNA conc. [ng/µl] [a]
Sediment				
(clean) ETC1	0.0711	0.0394	1.8	18
(clean) ETC2	0.0681	0.0388	1.8	17
(clean) ETC3	0.0728	0.0408	1.8	18
(mild) ETM1	0.0395	0.0235	1.7	10
(mild) ETM2	0.0449	0.027	1.7	11
(mild) ETM3	0.0373	0.0223	1.7	9
(DNAPL) ETD1	0.0453	0.0269	1.7	11
(DNAPL)ETD2	0.0437	0.0244	1.8	11
(DNAPL)ETD3	0.0446	0.0248	1.8	11

Source	Absorbance 260 nm	Absorbance 280 nm	Ratio 260/280	DNA conc. [ng/µl] [a]
Groundwater				
(clean) ETGWC	0.2221	0.2014	1.1	56
(mild) ETGWM	0.1711	0.1543	1.1	43
(DNAPL) ETGWD	0.2624	0.2315	1.1	66

[a] Extracted DNA was obtained in a total volume of 50 µl. All samples were diluted 1:5 before spectrophotometric analysis.

Table 3 summarizes the quantification of DNA obtained from the sediment and groundwater samples. Low amounts of high quality DNA were obtained from any of the 1 g sediment replicates (10-20 ng of DNA per µl). The least DNA was obtained from the sediments with "mild" contaminant concentrations. Groundwater yielded about 40-65 ng/µl of DNA in the final 50-µl volumes. The lowest concentration of DNA was obtained from the "mild" groundwater samples, which is consistent with the yields from the solid samples collected from the same location. The DNA extracted from the groundwater samples had a lower 260/280 ratio suggesting some protein contamination (Table 3), but no inhibitory effects on PCR amplification were observed.

Table 4 summarizes the results obtained with the group-specific primers. In the "clean" sediment samples (ETC-97-04) and the "DNAPL" (ETD-02-03B) sediment samples, 16S rRNA genes were readily amplified when community DNA was used as template for PCR with universal primers, suggesting that PCR-amplifiable DNA had been obtained. A weak signal was obtained with the DNA extracted from the "mild" (ETM-25-1A) sediment sample.

Table 4. PCR amplification with dechlorinator-targeted primer pairs using template DNA obtained from solid and groundwater (GW) samples.

	Target					
Sample	Universal	*Dehalo-coccoides*	*Desulfuro-monas*	*Dehalo-bacter*	*Desulfito-bacterium*	*Desulfo-monile*
Sediment						
ETC direct	+	-	-	-	-	-
ETC nested	+	-	-	-	-	-
ETM direct	w	-	-	-	-	-
ETM nested	w	+[a]	-	-	-	-
ETD direct	+	-	-	-	-	-
ETD nested	+	-	-	-	-	-
Groundwater						
ETGWC direct	+	-	-	-	-	-
ETGWC nested	+	+	-	-	-	+
ETGWM direct	+	-	-	-	-	-
ETGWM nested	+	+	-	-	-	-
ETGWD direct	+	-	-	-	-	-
ETGWD nested	+	+	-	-	-	-

(+) indicates that strong amplification occurred resulting in a clearly visible band following agarose gel electrophoresis.
(w) indicates a weak band was visible in agarose gels.
(-) indicates no visible band was obtained.
[a] One of three replicate samples tested positive.

PCR analysis with dechlorinator-targeted primer pairs using the extracted DNA as template (direct PCR) indicated the following:

- None of the five group-specific primers pairs yielded amplicons when DNA was used directly as template for PCR, suggesting that the target populations are absent or present in numbers too low to yield a signal with the direct PCR approach.

To avoid false-negative results, we used the more sensitive nested PCR approach. When 1:2 and 1:50 dilutions of the PCR product obtained with the universal primer pair was used as template in a second round of PCR (i.e., nested PCR) with the group-specific primer pairs, the following results were obtained (summarized in Table 4).

- The nested PCR approach identified *Dehalococcoides* populations in one of three replicate sediment extractions performed for the "mild" samples (ETM-25-1A).
- The nested PCR approach identified *Dehalococcoides* populations in all three groundwater samples (ETGWC-97-04, ETGWM-25-1A and ETGWD-02-03B).
- The nested PCR approach identified *Desulfomonile* populations in the "clean" groundwater (ETGWC-97-04).
- The nested PCR approach did not identify *Desulfuromonas*, *Dehalobacter* or *Desulfitobacterium* populations in any of the sediment or groundwater samples.

Apparently, *Dehalococcoides* species were present in the site samples. *Dehalococcoides* species, however, were detected only with the sensitive nested PCR approach, suggesting that *Dehalococcoides* populations are present in the groundwater in low numbers.

Another sensitive, nucleic acid-based approach to detect specific target sequences is RTm PCR. RTm PCR has the added advantage of providing quantitative information, and this approach was used to quantify *Dehalococcoides* 16S rRNA genes, and the *bvcA* and *tceA* genes (12). Total 16S rRNA gene copy numbers were also determined using a modified protocol of Harms et al. (2). The *Dehalococcoides* 16S rRNA gene targeted primers and probe set quantifies total *Dehalococcoides* cells in a given sample, assuming one 16S rRNA gene copy per genome (3). The approach does not distinguish between *Dehalococcoides* subgroups, nor does it predict a particular dechlorinating activity. The *tceA* gene-targeted primers and probe quantify the number of copies of the *Dehalococcoides*-derived *tceA* gene that is known to be involved in TCE and *cis*-DCE dechlorination to VC. The *bvcA* gene targeted primers and probe quantify the number of copies of the gene that was recently found to be involved with the dechlorination of VC to ethene in *Dehalococcoides* sp. strain BAV1 (5). Both, the *tceA* and the *bvcA* genes are present as single copies on the *Dehalococcoides* genome, indicating that the number of genes detected equals the number of *Dehalococcoides* cells present in the sample.

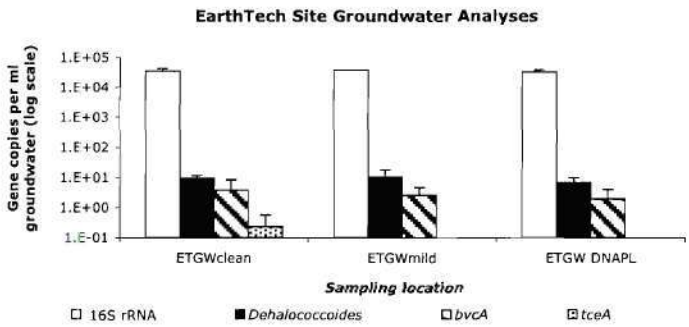
RTm PCR confirmed the nested PCR results, and detected *Dehalococcoides* in all groundwater samples. RTm PCR analysis also showed that 25-40% of the *Dehalococcoides* populations possess a gene similar to *bvcA*, the gene coding for the vinyl chloride reductive dehalogenase in *Dehalococcoides* sp. strain BAV1 (5). The numbers of *Dehalococcoides* cells in all samples were low, and near the detection limit of the RTm approach (Table 5).

Table 5. Quantification of *Dehalococcoides*-specific gene targets in groundwater samples.

	Gene copies per ml of groundwater			
	Bacterial 16S rRNA	*Dehalococcoides*	*bvcA*	*tceA*
ETGW clean	34,300	9.43	3.79	0.23
ETGW mild	38,300	10.39	2.55	-
ETGW DNAPL	31,900	6.87	1.91	-

There was very little difference between the three sampling locations, in terms of *Dehalococcoides* abundance. For clearer presentation, the results presented in Table 5 are visualized in the form of a bar graph (Figure 1). *Dehalococcoides* populations represent 0.3% of the total bacterial community.

Figure 1.
Total bacterial 16S rRNA gene copies and *Dehalococcoides* gene copies per ml of groundwater. Note that the y-axis has a logarithmic scale.



Report 2: Microbial Analysis of Microcosms After 206 Days of Incubation

- Performed by Kirsti Ritalahti, Ph.D.

OBJECTIVE

To determine the impact of enrichment conditions on indigenous, reductively dechlorinating bacteria, molecular analyses were performed on select microcosms following a 206-day incubation with C-56 (± lactate) and C-58 (± lactate). The microcosms used for nucleic acid extraction are summarized in Table 6 and highlighted in Table 13.

Table 6. Microcosms sacrificed and analyzed with nucleic acid-based tools in Nov. 2004

Sample	Analyzed	Treatment	Electron Donor	Materials Extracted
ET1-G	14/Nov/04	Groundwater, no C-56	None	0.7g solids, 5.5 ml supernatant
ET2-G	14/Nov/04	Groundwater, no C-56	None	0.7g solids, 5.5 ml supernatant
ET3-GL	14/Nov/04	Groundwater, no C-56	5 mM lactate	0.7g solids, 5.5 ml supernatant
ET4-ML	14/Nov/04	Medium, 5 ppM C-56	5 mM lactate	0.7g solids, 5.5 ml supernatant
ET5-ML	14/Nov/04	Medium, 5 ppM C-56	5 mM lactate	0.7g solids, 5.5 ml supernatant
ET6-M	14/Nov/04	Medium, 5 ppM C-56	None	0.7g solids, 5.5 ml supernatant
ET7-M	14/Nov/04	Medium, 5 ppM C-56	None	0.7g solids, 5.5 ml supernatant

Nomenclature indicates the vial number assigned to each of the sacrificed microcosms: ET1 to ET7, followed by the treatment type, groundwater (G), mineral salts medium (M), and/or lactate amendment (L). The term 'solid (s)' is used to specify that DNA was obtained from the solid fraction.

METHODS

DNA isolation. Seven microcosms were sacrificed for microbial community analyses following a 206-day incubation period. Microcosm supernatant (5.5 ml) was poured off into a 15-ml falcon tube and the cells were collected by centrifugation (8,000 x g, 30 min). DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, California) with modifications as described previously (11). The DNA was eluted in 200 µl Tris buffer (0.1 mM Tris, pH 8.5). DNA was also extracted from 0.7 g samples of microcosm sediment (solids). Solids were aseptically transferred to MoBio Soil Extraction vials, and extracted as per manufacturer recommendations (MoBio Laboratories Inc., Carlsbad, CA). The DNA was eluted in 50 µl Tris Buffer (0.1 mM Tris, pH 8.5) and then quantified by spectrophotometry at a wavelength of 260 nm.

Detecting and quantifying known dechlorinating bacterial groups and indicator genes. Qualitative and quantitative PCR was performed as described above for the aqueous and sediment samples. For nested PCR, 1:10 and 1:50 dilutions of the PCR product obtained with the universal primer pair was used as template in a second round of PCR (i.e., nested PCR) with the group-specific primer pairs.

RESULTS

Table 7 summarizes the quantification of DNA from the microcosms following a 206-day incubation period. High quality DNA was obtained from all of the 0.7 g sediment samples. While more DNA was obtained from the liquid fraction, this was not of as high quality as the DNA from the sediment.

Table 7. DNA extracted from microcosms.

Source	Absorbance 260 nm	Absorbance 280 nm	Ratio 260/280	DNA conc. [ng/µl] [a]
ET1-G solids	0.0168	0.0105	1.6	4
ET2-G solids	0.0288	0.0184	1.6	7
ET3-GL solids	0.0234	0.0139	1.7	6
ET4-ML solids	0.0569	0.0413	1.4	14
ET5-ML solids	0.0577	0.0413	1.4	14
ET6-M solids	0.0205	0.0126	1.6	5
ET7-M solids	0.0364	0.0197	1.8	9
ET1-G supernatant	0.0814	0.0681	1.2	20
ET2-G supernatant	0.1761	0.1469	1.2	44
ET3-GL supernatant	0.1758	0.1482	1.2	44
ET4-ML supernatant	0.1638	0.1369	1.2	41
ET5-ML supernatant	0.3645	0.3057	1.2	91
ET6-M supernatant	0.2198	0.1841	1.2	55
ET7-M supernatant	0.1059	0.0872	1.2	26

[a] Extracted DNA was obtained in a total volume of 50 µl. All samples were diluted 1:5 before spectrophotometric analysis.

Universal bacterial 16S rRNA gene-targeted primers generated the appropriately sized product (~1,500 bp) for all DNA samples (Table 8). *Dehalococcoides* populations were detected using direct PCR in enrichment cultures ET1-G, ET2-G and ET7-M. No other bacterial groups (*Desulfuromonas*, *Dehalobacter*, *Desulfitobacterium*, or *Desulfomonile*) were detected in any of the microcosms using the direct PCR approach.

Table 8. PCR amplification of microcosm DNA with dechlorinator-targeted primer pairs.

Sample	Target					
	Universal	*Dehalo-coccoides*	*Desulfuro-monas*	*Dehalo-bacter*	*Desulfito-bacterium*	*Desulfo-monile*
ET1-G solids	+	+*	-	+	-	-
ET2-G solids	+	+*	-	+	-	-
ET3-GL solids	+	-	-	-	-	-
ET4-ML solids	+	+	-	-	-	-
ET5-ML solids	+	-	-	-	-	-
ET6-M solids	w	+	-	+	-	-
ET7-M solids	+	+*	-	+	-	-
ET1-G supernatant	+	+	-	+	-	-
ET2-G supernatant	+	+	-	+	-	-
ET3-GL supernatant	w	-	-	-	-	-
ET4-ML supernatant	+	-	-	-	-	-
ET5-ML supernatant	+	-	-	-	-	-
ET6-M supernatant	+	+	-	+	-	-
ET7-M supernatant	+	+	-	+	-	-

(+) indicates that strong amplification occurred resulting in a clearly visible band following agarose gel electrophoresis.
(w) indicates a weak band was visible in agarose gels.
(-) indicates no visible band was obtained.
* Indicates amplification product obtained in direct PCR.

To avoid false-negative results, we used the more sensitive nested PCR approach. In the cases where the nested PCR was positive, the 1:10 and 1:50 dilutions both yielded the expected amplicon. Table 8 summarizes the results obtained with the group-specific primers using the direct and nested PCR approach. The results of the molecular analyses following a 206-day incubation period indicate the following:

- No dehalogenating bacterial populations were identified using nested PCR in the sediment of any of the lactate amended samples with one exception: ET4-GL had a positive signal for *Dehalococcoides*.
- The nested PCR approach identified *Dehalococcoides* populations in the sediment and supernatant fractions of ET1-G, ET2-G, ET4-GL, ET6-M and ET7-M.
- The nested PCR approach identified *Dehalobacter* populations in both the sediment and supernatant of ET1-G, ET2-G, ET6-M and ET7-M.

Quantitative RTm PCR was used to estimate total bacterial 16S rRNA gene copies, as well as total *Dehalococcoides* 16S rRNA genes, and the *bvcA* and *tceA* genes. The RTm PCR approach further verified the results of the nested PCR approach in detecting *Dehalococcoides* (Table 9).

Table 9. Quantification of total bacterial 16S rRNA genes and *Dehalococcoides*-specific gene targets in microcosm solids.

	Gene copies per ml of solids			
	Bacterial 16S rRNA	*Dehalococcoides* 16S rRNA	*bvcA*	*tceA*
ET1-Gs	1.0×10^7	6.7×10^6	4.5×10^4	2.8×10^5
ET2-Gs	1.4×10^7	9.5×10^6	3.5×10^4	9.5×10^5
ET3-GLs	2.3×10^7			
ET4-MLs	2.8×10^8			
ET5-MLs	1.0×10^7			
ET6-Ms	5.7×10^5	6.1×10^4	2.3×10^3	6.9×10^4
ET7-Ms	1.2×10^8	2.4×10^6	2.9×10^3	4.2×10^5

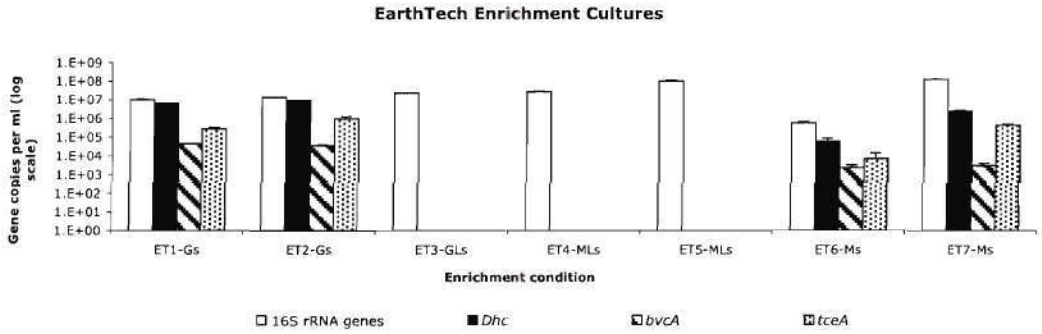
There was very little difference between the three sampling locations, in terms of *Dehalococcoides* abundance.

- All samples contained 10^7 to 10^8 total bacterial 16S rRNA genes per gram of solids.
- The solid samples that had tested positive for *Dehalococcoides* (i.e., ET1-Gs, ET2-Gs, and ET7-Ms) with the direct PCR approach could be quantified with the RTm PCR approach. In addition, ET6-Ms, which tested positive for *Dehalococcoides* with the nested PCR approach and both the 1:10 and 1:50 dilutions of the 16S rRNA genes, could also be quantified.
- Sample ET4-ML, which tested positive with the nested PCR with *Dehalococcoides*-targeted primers and the 1:10 dilution of community 16S rRNA genes, did not contain quantifiable numbers of *Dehalococcoides* cells.
- No *Dehalococcoides* 16S rRNA genes, nor reductive dehalogenase genes, were quantified in ET3-GL, ET4-ML or ET5-ML (enrichments with lactate).

The reason why *Dehalococcoides* cell numbers did not increase in the lactate amended microcosms was unexpected. The growth requirements of *Dehalococcoides* species are poorly understood, though it is known that substrate concentrations and pH affect growth. Hence, it is possible that the high substrate concentration and a drop in pH to below 6.5 following lactate fermentation limited growth of *Dehalococcoides* species.

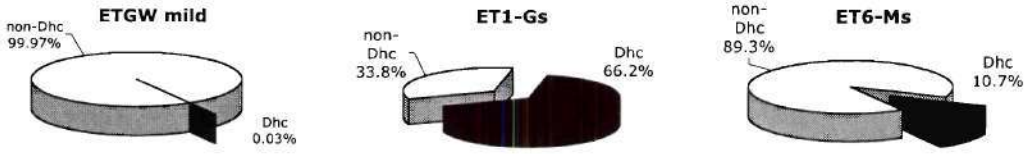
For clearer presentation, the results presented in Table 9 are visualized in form of a bar graph (Figure 2).

Figure 2. Total bacterial 16S rRNA genes and *Dehalococcoides* gene copies per gram of microcosm solids. Note that the y-axis has a logarithmic scale.



Dehalococcoides populations increased significantly as a proportion of the total bacterial 16S rRNA gene abundance in the microcosms without lactate amendment. In the initial groundwater from the "mild" location, only 0.03% of the microbial community was composed of *Dehalococcoides* populations. No *Dehalococcoides* were detected in any of the original sediment samples. After enrichment in groundwater microcosms, *Dehalococcoides* comprised nearly 66% of the total bacterial community of the sediment portion (ET1-G and ET2-G). In mineral salts medium, *Dehalococcoides* increased to 2-10% of the bacterial community (ET6-M, ET7-M). Figure 3 shows a representative microcosm for each treatment.

Figure 3. Distribution of *Dehalococcoides* genes as a fraction of the total bacterial community. Shown below are: the initial groundwater sample from the 'mild' contaminated location, and the solids from microcosms following enrichment in groundwater (ET1-Gs) and after enrichment in mineral salts medium (ET6-Ms).



Report 3: Contaminant Analysis of Sediment Materials and Groundwater

- Performed by Qingzhong Wu, Ph.D.

OBJECTIVE

Determine the concentrations of hexachlorocyclopentadiene (C-56) and octachlorocyclopentene (C-58) in groundwater and sediment samples collected at the former Occidental Chemical Corporation (OCC) facility in Montague, Michigan. The samples were collected in January 2004 and shipped on blue ice in a cooler to Georgia Tech.

The soil cores carried labels 'clean', 'mild', and 'DNAPL', and two bottles of groundwater from each sampling site were supplied. Duplicate samples from each container were analyzed for C-56 and C-58 (i.e., a total of 12 groundwater and 12 sediment samples were analyzed).

METHODS

Groundwater (4 ml) and sediment samples (4 g) were transferred to 20-ml glass vials and closed with screw caps fitted with Teflon-lined septa. Distilled water (6 ml) was added to the vials containing solids before 2 ml of hexane were added to all vials. The vials were shaken overnight on rotary shaker at 250 rpm. One ml of the organic layer (hexane phase) was transferred to 2-ml glass autosampler vials and immediately closed with Teflon-lined screw caps. The vials were transferred to a Hewlett Packard 7683 autosampler (1 µl injection volume), and analyzed with a Hewlett Packard 6890 series gas chromatograph equipped with a HP-624 capillary column (60 m length, 0.32 mm inner diameter, 1.8 µm film thickness), and a Ni^{63} micro electron capture detector. The injection temperature was 235°C and the detector temperature was 300°C. The oven temperature was 80°C and held 3 min, then raised to 200°C at a rate of 10°C per min, held at 200°C for 2 min, before the temperature was increased to 250°C for 2 min.

Fourteen-point calibration curves were generated with C-56 and C-58 standards purchased from Sigma-Aldrich (catalog numbers 40051 and 8370142, respectively) diluted in hexane. The calibration curve covered a range of 0.0016-13.2 µg/ml of C-56 and C-58. Standards (1 ppm of C-56 and C-58) were included each time samples were analyzed. The method used followed essentially the procedures described in EPA SW846 for sample preparation, extraction, and analysis. Possible differences include the size of the extraction vials, the choice of solvents used for the extraction, and the use of GC/MS for peak identification.

RESULTS

Sample concentrations were all within the linear range of the calibration curve, except for one of the replicate samples from the DNAPL sediment, as summarized in Table 10.

Table 10. Concentrations of C-56 and C-58 in groundwater and sediments supplied by EarthTech in January 2004.

Sample ID	**Groundwater mg/l** [a]		**Sediment mg/kg** [a]		
	C-56	C-58	Label [b]	C-56	C-58
Clean	0.0004[c]	0.0005[c]	T20-B20.5	1.07	5.634
	0.0007	0.0007	T23-B24	0.084	0.5
Mild	0.014	0.0002	T20-B20.5	0.4	2.87
	0.03	0.0006	T23-B24	0.26	1.79
DNAPL	0.24	0.0045	T18-B18.5	0.002	0.71
	0.23	0.003	T23-B24	98.1[c]	133.92[c]

[a] Data shown are averaged from two independently extracted samples collected from the same container.

[b] Indicates the label on the soil cores.

[c] Indicates that the concentration of C-56 or C-58 was outside the linear range of the standard curve.

Report 4: Evaluation of Anaerobic Microbial Degradation of Hexachlorocyclopentadiene (C-56) and Octachlorocyclopentene (C-58) in Sediment Materials and Groundwater

- Performed by Qingzhong Wu, Ph.D.

OBJECTIVE

Investigate the anaerobic microbial degradation/transformation of hexachlorocyclopentadiene (C-56) and octachlorocyclopentene (C-58) in groundwater and sediment samples collected at the former Occidental Chemical Corporation (OCC) facility in Montague, Michigan.

METHODS

Preparation of microcosms. EarthTech personnel collected groundwater and sediment samples in January 2004. The samples were shipped to Georgia Tech on blue ice in a cooler. Microcosms were prepared in an anoxic chamber filled with N_2/H_2 (97/3, vol/vol). Microcososms were prepared at two concentrations of C-56 and C-58: below and above the solubility of these compounds in water. Because the concentration of C-56 in the sediment materials labeled as 'mild T20-B20.5' and 'mild T23-24' (see Table 10 for concentrations of C-56 and C-58 in the sediment materials) indicated that no C-56 DNAPL was present, the 'mild' sediments were used for establishing microcosm. The sediments were physically mixed with in sterile Mason jars inside the anoxic chamber. About 4 grams of the sediment materials were placed in sterile 20-ml glass vials. Microcosms were established with 'clean' groundwater (pH 7.1) obtained from the same locations where the sediments were collected. In addition, microcosms were established with anoxic, bicarbonate-buffered mineral salts medium (pH 7.1). The medium was prepared as described previously except that resazurin was omitted (7, 10). The medium contained (per liter): 0.5 g of $MgCl_2$ x 6 H_2O, 1 g of NaCl, 0.2 g of KH_2PO_4, 0.3 g of NH_4Cl, 0.3 g of KCl, 0.015 g of $CaCl_2$ x 2 H_2O, 2.52 g of $NaHCO_3$, 0.035 g of L-cysteine, 0.048 g of Na_2S x 9 H_2O, 1 ml trace element solution A, 1 ml trace element solution B, and 1 ml vitamin solution (8). The final volume in each vial was 10 ml. Microcosms were also established in 60-ml glass serum bottles. Each 60-ml bottle contained 12 grams of sediment material and groundwater or mineral salts medium were added to final volumes of 30 ml. C-56 dissolved in methanol was added to each vial to yield final concentrations of 1 mg/l in non-DNAPL microcosms. Lactate (5 mM) was added as a source of reducing equivalents. Lactate is readily fermented by ubiquitous soil microorganisms to propionate, acetate, and hydrogen, and thus, has been an electron donor of choice for field-scale bioremediation approaches (6).

Literature references (14) list the aqueous solubility of C-56 at ~1 mg/l. Concentrations of 3 mg/l C-56 were established in another set of vials to yield microcosms containing free phase product (i.e., dense, non-aqueous liquid or DNAPL). C-56 was added from a methanol stock solution (10 mg/ml), and the total amount of methanol added to a microcosm did not exceed 0.1% (vol/vol). Since information on the aqueous solubility of C-58 was not available, we assumed that the solubility of C-58 is similar to that of C-56. No C-58 was added to non-DNAPL microcosms because contaminant concentrations in the sediments used to initiate the microcosms ranged form 1.8-2.9 mg/kg (Table 10). To establish microcosms containing free phase product, C-58 was added to a final concentration of 3 mg/l. However,

the concentration of C-58 in the analyzed microcosms (Table 12, and Table 13) was actually much higher at time zero than in the original sediment samples as detected with GC/ECD (Table 10), indicating an uneven distribution of C-58 in the sediment material.

Dr. Löffler's laboratory developed a chloroethenes-detoxifying consortium exhibiting robust reductive dechlorinating activity. This culture has been successfully used in bioaugmentation approaches (6). We tested the ability of this culture to promote enhanced reductive dechlorination of the target compounds C-56 and C-58 in microcosms (bioaugmentation). Microcosms undergoing bioaugmentation were inoculated with the PCE-to-ethene-dechlorinating consortium. Treatment conditions are shown in Table 11. To test the ability of a PCE-to-ethene-dechlorinating consortium to promote enhanced reductive dechlorination of the target compounds C-56 and C-58, 0.2 ml or 0.6 ml of the reductively dechlorinating consortium were added to the 20-ml and 60-ml microcosms, respectively (i.e., 2% inoculum by volume). Lactate (5 mM) was added to the bioaugmented microcosms as electron donor. Additional microcosms were amended with 5 mM lactate to explore the ability of native microorganisms to transform C-56 and C-58 (i.e., biostimulation). As controls, and also to monitor dechlorination of chloroethenes in these microcosms, PCE and *cis*-DCE were added to selected replicate microcosms by syringe to final aqueous concentrations of 0.29 mM and 0.57 mM, respectively.

The vessels were closed with new Teflon-lined butyl rubber stoppers and incubated without agitation at 22°C in the dark with the stoppers up. Controls included killed microcosms (autoclaved at 121°C for one hour on two consecutive days) to assess abiotic and sorptive losses of substrates and products. Controls also included microcosms that did not receive lactate as a source of carbon and reducing equivalents or chlorinated electron acceptors to assess any background metabolic activity. Organic acids were analyzed by high-performance liquid chromatography using a Waters Breeze system (Waters, Milford, Mass) equipped with a Waters 2487 dual-wavelength absorbance detector and a Waters 717 plus autosampler (8). Table 11 summarizes the microcosms and the different treatment regimes that were established.

Legend to Table 11

[a] C-56 was added into non-DNAPL microcosms and DNAPL microcosms to final concentration of 1 and 3 mg/l, respectively. C-58 was added into C-58 DNAPL microcosms at final concentration of 3 mg/l, and no C-58 was added into non-DNAPL microcosms. The sediment samples labeled as 'mild T20-B20.5' and 'mild T23-24' were used to prepare all microcosms. For bioaugmentation, 10- and 30-ml microcosms were inoculated with 0.2 ml or 0.6 ml of a mixed culture capable of dechlorinating chloroethenes, respectively. Lactate (5 mM) was added to select microcosms as an electron donor as indicated in the table.

[b] Entire microcosms were sacrificed for the analysis of C-56 and C-58.

[c] DNAPL indicates that the final concentrations of C-56 or C-58 in these microcosms following amendment with C-56 and C-58 were about 3 mg/l for both compounds.

[d] Culture liquid (1 ml) was withdrawn for C-56 and C-58 analysis (1 ml was extracted with 2 volumes of hexane) and organic acid analysis (analyzed in the aqueous phase).

[e] x 2 indicates two sets of microcosms were established (i.e., C-56- and C-58-amended microcosms).

Table 11. Microcosms and treatment regimes to test transformation of C-56 and C-58 [a].

Treatment	Bioaugmentation	Electron acceptor	e⁻ donor	Number of microcosms	Time of analysis
in 10-ml microcosms [b]		**144 microcosms**			
with groundwater		14 microcosms			
live	No	C-56 & C-58	lactate	5	at 0, 28, 56, 84, & 112 days
live	Yes	C-56 & C-58	lactate	4	at 28, 56, 84, & 112 days
live	No	C-56 & C-58	none	2	at 56 & 112 days
killed	Yes	C-56 & C-58	lactate	3	at 0, 56 & 112 days
with medium		28 x 2 microcosms[e]			
live	No	C-56 or C-58	lactate	5 x 2 (duplicate)	at 0, 28, 56, 84, & 112 days
live	Yes	C-56 or C-58	lactate	4 x 2 (duplicate)	at 28, 56, 84, & 112 days
live	No	C-56 or C-58	none	2 x 2 (duplicate)	at 56 & 112 days
killed	Yes	C-56 or C-58	lactate	3 x 2 (duplicate)	at 0, 56 & 112 days
with medium and DNAPL[c]		28 x 2 microcosms[e]			
live	No	C-56 DNAPL or C-58 DNAPL	lactate	5 x 2 (duplicate)	at 0, 28, 56, 84, & 112 days
live	Yes	C-56 DNAPL or C-58 DNAPL	lactate	4 x 2 (duplicate)	at 28, 56, 84, & 112 days
live	No	C-56 DNAPL or C-58 DNAPL	none	2 x 2 (duplicate)	at 56 & 112 days
killed	Yes	C-56 DNAPL or C-58 DNAPL	lactate	3 x 2 (duplicate)	at 0, 56 & 112 days
with medium and PCE or DCE		9 x 2 microcosms[e]			
live	No	PCE or *cis*-DCE	lactate	x 3 (triplicate)	at 0, 28, 56, 84, & 112 days
live	Yes	PCE or *cis*-DCE	lactate	x 3 (triplicate)	at 0, 28, 56, 84, & 112 days
killed	No	PCE or cis-DCE	lactate	x 3 (triplicate)	at 0, 28, 56, 84, & 112 days
in 30-ml microcosms [d]		**12 microcosms**			
with groundwater		4 microcosms			
live	No	C-56 & C-58	lactate	x 2 (duplicate)	at 28, 56, 84, & 112 days
live	Yes	C-56 & C-58	lactate	x 2 (duplicate)	at 28, 56, 84, & 112 days
with medium and DNAPL[c]		4 x 2 microcosms[e]			
live	No	C-56 DNAPL or C-58 DNAPL	lactate	x 2 (duplicate)	at 0, 28, 56, 84, & 112 days
live	Yes	C-56 DNAPL or C-58 DNAPL	lactate	x 2 (duplicate)	at 0, 28, 56, 84, & 112 days
Total		**156 microcosms**			

RESULTS

The analyses indicated that the average C-56 and C-58 concentrations in the 'mild T20-B20.5' and 'mild T23-B24' sediment used for preparation of microcosms were 0.33 mg/kg ((0.4 + 0.26) ÷ 2 mg/kg) and 2.33 ((2.87 + 1.79) ÷ 2 mg/kg), respectively (see Table 10). Tables 12 and 13 show the concentrations of C-56 and C-58 in the microcosms over time. Concentrations of C-56 and C-58 varied considerably between microcosms analyzed on day zero. A possible explanation for the variations is an uneven distribution of the contaminants and the presence of C-58 DNAPL in the sediment materials used for microcosm setup. Despite thoroughly mixing the sediment materials prior to microcosm setup, an even contaminant distribution was apparently not achieved before initiating the microcosms.

Table 12. Change in C-56 and C-58 concentrations (mg/l) in 30-ml microcosms [a]

Treatment	0 day		28 days		56 days[c]	
	C-56	C-58	C-56	C-58	C-56	C-58
with groundwater						
live w/ lactate	-[b]	-[b]	0.07	0.72	0.06 (97)	0.84 (93)
live w/ lactate & bioaugmentation	-[b]	-[b]	0.09	1.02	0.06 (97)	0.85 (92)
with medium and DNAPL						
live w/ lactate	2.93	8.91	0.07	0.85	0.07 (97)	1.00 (91)
live w/ lactate & bioaugmentation	3.26	10.92	0.07	0.88	0.06 (97)	0.83 (93)
live w/ lactate	0.98	12.35	0.07	0.99	0.06 (97)	0.95 (92)
live w/ lactate & bioaugmentation	1.05	12.81	0.07	0.81	0.07 (97)	1.00 (91)

[a] data shown were averaged from duplicate samples. No data were collected after 84 and 112 days as originally planed (see Table 11) because microcosms were amended with additional C-56 and C-58 after 81 days of incubation (= time zero in Table 15). These microcosms were used as biotic controls in Report 5: abiotic investigations.

[b] no samples were analyzed

[c] data in parentheses represent percentage of removal of C-56 or C-58 after 56 days of incubation. Initial concentrations of C-56 and C-58 were 2.06 and 11.25 mg/ml, respectively (calculated from averaged concentrations of C-56 and C-58 at time zero).

As shown in Table 13, concentrations of C-56 and C-58 in all autoclaved microcosms were very low (< 0.02 ppm), suggesting that autoclaving resulted in a decrease in C-56 and C-58 concentrations. Chloride release in live and killed microcosms could not be quantified and compared due to the high amount of background chloride present in mineral medium used in the experiment.

Table 13. Change in the concentration of C-56 and C-58 (mg/l) in 10-ml microcosms [a]. Shaded boxes indicate microcosms that were analyzed with nucleic acid-based approaches following the 206-day incubation period (see above).

Treatment	0 day		56 days		95 days		206 days[c]	
	C-56	C-58	C-56	C-58	C-56	C-58	C-56	C-58
with groundwater[b]								
live w/o lactate	-	-	-	-	-	-	0.07 (95)	0.09 (99)
live w/ lactate	0.86	4.95	**0.05**	**0.78**	-	-	0.01 (99)	0.08 (99)
live w/ lactate & mixed culture	-	-	**0.05**	**0.66**	**0.02**	**0.17**	0.01 (99)	0.17 (99)
killed w/ lactate & mixed culture	**0.01**	**0.02**	**0.00**	**0.00**	-	-	**0.00 (100)**	**0.01 (100)**
with medium								
C-56								
live w/o lactate	-	-	**0.08**	**1.26**	**0.02**	**0.17**	0.05 (97)	0.9 (94)
live w/ lactate	1.09	19.92	0.20	3.08	0.03	0.04	0.05 (97)	0.79 (95)
live w/ lactate & mixed culture	-	-	0.09	1.46	0.06	0.81	0.05 (97)	1.17 (92)
killed w/ lactate & mixed culture	0.00	0.06	**0.01**	**0.01**	**0.00**	**0.00**	0.00 (100)	0.01 (100)
C56 (DNAPL)								
live w/o lactate	-	-	**0.20**	**2.51**	**0.03**	**0.39**	0.01 (99)	0.19 (99)
live w/ lactate	3.10	18.14	0.09	1.71	0.04	0.53	0.02 (99)	0.21 (99)
live w/ lactate & mixed culture	-	-	0.12	1.73	0.02	0.30	0.04 (97)	0.52 (97)
killed w/ lactate & mixed culture	0.00	0.02	0.00	0.00	-	-	0.00 (100)	0.01 (100)
C-58								
live w/o lactate	-	-	0.06	1.11	-	-	0.04 (97)	0.48 (97)
live w/ lactate	0.90	13.66	0.05	1.18	0.03	0.44	0.06 (96)	0.45 (97)
live w/ lactate & mixed culture	-	-	0.04	1.20	0.03	0.39	0.03 (98)	0.43 (97)
killed w/ lactate & mixed culture	0.00	0.01	0.00	0.01	-	-	0.00 (100)	0.01 (100)
C58 (DNAPL)								
live w/o lactate			**0.09**	**1.20**	**0.08**	**1.01**	0.02 (99)	0.25 (98)
live w/ lactate	1.39	19.39	0.06	0.77	0.02	0.31	0.03 (98)	0.43 (97)
live w/ lactate & mixed culture			0.09	1.05	0.03	0.37	0.02 (99)	0.31 (98)
killed w/ lactate & mixed culture	0.00	0.01	0.00	0.00			0.00 (100)	0.01 (100)

[a] Entire microcosms were sacrificed for the analysis of C-56 and C-58. No samples were analyzed after 28 days as originally planed (see Table 11). The original sampling schedule changed, and samples were analyzed after 95 and 206 days, and not at after 84 and 112 days as outlined in Table 11.

[b] A minus (-) sign indicates that no samples were analyzed; number in bold represent data from single samples and number in normal font represent averaged data from duplicate or triplicate samples.

[c] Data in parentheses represent percentage of removal of C-56 or C-58 after 206 days of incubation. Initial concentrations of C-56 and C-58 were 1.468 and 15.212 mg/ml, respectively (calculated from averaged concentrations of C-56 and C-58 at time zero).

Although it is difficult to prove that evaporative loss is negligible, we believe that the loss of C-56 and C-58 through the septum is not a significant sink. We routinely perform experiments with chlorinated ethenes using the same vessels and septa, and we carefully evaluated loss in these systems. Assuming the vials were sealed properly, what we have done, loss of volatile organics through the septum is negligible. Even under increased pressure during the autoclave cycle, the seal prevents leakage of volatiles from the vials. We tested this repeatedly with chlorinated ethenes, and >95% of the initial mass added is recovered following autoclaving.

Sorption is another possible sink for chloroorganic compounds. Sorption to the glass walls

was excluded as a major sink because we performed organic solvent (hexane) extractions. We determined that the extraction procedure recovers >95% of the C-56 or C-58 added to a vial when the procedure was performed without prolonged incubation. Sorption of C-56 or C-58 to the the septum is another possible sink. We used new Teflon-lined septa that had not been punctured for all experiments. The vials were incubated with the stopper up, and the headspace concentrations of C-56 or C-58 are very low, suggesting that sorption through the Teflon lining into the rubber is unlikely to be a major sink for the target compounds. In summary, these findings do not support sorption as a dominant mechanism to drive the significant decrease of C-56 and C-58 observed in our experiments.

Transformation of the target compounds during the autoclave cycle, rather than evaporative or sorptive losses, are the most probable cause for the decrease in concentration (discussed in more detail below). During the 206-day incubation period, the concentrations of C-56 and C-58 decreased in all live microcosms. No degradation products were identified by gas chromatography in the headspace or the hexane extracts of groundwater and solids from the microcosms. Methane was observed in the headspace of all live microcosms, indicating methanogenic activity, and that anaerobic conditions conducive for reductive dechlorination activity were established (0.1 ml of headspace sample analyzed using GC/FID, methane formation was not quantitatively monitored). Lactate was analyzed in 30-ml microcosms after 7 - 11 and 206 days of incubation. Lactate concentrations in samples from these microcosms continually decreased after 7-11 days, and no lactate was detected after 206 days in any of the 30-ml microcosms.

In microcosms supplemented with PCE, dechlorination to *cis*-DCE only occurred in the microcosms amended with the PCE-to-ethene-dechlorinating inoculum. No ethene formation, however, has occurred to date, suggesting an inhibitory effect of the sediment materials on reductive dechlorination beyond *cis*-DCE. No chloroethene reductive dechlorination occurred in microcosms that were not amended with the dechlorinating consortium, suggesting that PCE-dechlorinating bacteria were not associated with the sediment material and groundwater. The results are summarized in Table 14.

Table 14. End products in microcosms amended with PCE and *cis*-DCE after 70 days.

Treatment	End product (70 days)
PCE + lactate	
live	PCE
killed	PCE
live + inoculum	*cis*-DCE[a]
cis-DCE + lactate	
live	*cis*-DCE
killed	*cis*-DCE
live + inoculum	*cis*-DCE

[a] PCE (0.29 mM) was completely dechlorinated to *cis*-DCE after 70 days of incubation. Data are averaged from triplicate microcosms.

Report 5: Abiotic investigations to determine the fate C-56 and C-58

- Performed by Qingzhong Wu, Ph.D.

OBJECTIVE

To further explore the disappearance of C-56 and C-58 in the absence of biological activity, experiments were initiated to determine the fate of the target compounds under abiotic conditions.

METHODS

Glass vials (20 ml nominal capacity) filled with freshly prepared minimal medium (identical to the medium used for the biotic experiments) or distilled water were autoclaved at 121°C for 30 minutes and cooled to room temperature (20-25°C) before C-56 and C-58 were added. The vials were incubated at 4°C and 22°C in the dark. An additional set of vials was incubated at room temperature (~15 - 25°C), exposed to light (i.e., on a window sill exposed to sunlight and to ambient room light). Following the observation that the target compound concentrations had decreased significantly in all microcosms (Table 12), 30-ml microcosms established with groundwater and medium in April of 2004 with C-58 DNAPL were amended with additional C-56 and C-58 after the original 81 days of incubation to serve as biotic controls. The term "microcosms" indicates that sediment and microorganisms were added to groundwater or minimal medium. The 30-ml microcosms were incubated at 22°C in the dark.

Concentrations of C-56 and C-58 in headspace samples were estimated by analyzing the peak areas of C-56 and C-58, using standard curves generated for C-56 and C-58 as described in the methods section of Report 3.

RESULTS

Table 15 summarizes the results of these experiments. The concentrations of C-56 and C-58 decreased in the absence of microbes under sterile conditions, indicating that abiotic mechanisms contribute to the decrease in C-56 and C-58. Although more detailed studies are needed to explore the abiotic decline of the target compounds, these initial studies suggest that the fate of C-56 and C-58 is affected by temperature and light exposure. Since no new peaks were identified in hexane extracts using GC/ECD analysis, we analyzed headspace samples of microcosms in which C-56 and C-58 had disappeared to identify possible transformation products. Using GC/FID analysis, no peaks were observed in headspace of the microcosms or the water-containing vials amended with C-56 and C-58. Using GC/ECD, small peaks of C-56 (0.02 ppm and 0.08 ppm in headspace samples incubated at 4 and 22°C, respectively) and C-58 (0.004 ppm and 0.01 ppm in headspace samples incubated at 4 and at 22°C, respectively) were observed in vials without solids. Chloride in these samples was not analyzed because of the high chloride background present in the mineral medium. Hexane extracts were not analyzed using GC/FID.

Table 15. Comparison of change in concentrations of C-56 and C-58 in water, minimal medium, and microcosms (mg/l) [a]

Treatment	0 day		10 days[c]		0 days		16 days [c]	
	C-56	C-58	C-56	C-58	C-56	C-58	C-56	C-58
	in minimal medium				in water			
22°C at dark	0.9	3.04	0.07 (92)	0.78 (74)	2.25	3.28	0.59 (74)	1.03 (54)
4°C at dark[b]	1.1	3.32	0.12 (89)	2.4 (28)	2.84	3.64	1.46 (49)	1.92 (32)
room temperature w/ light	1	3.19	0 (100)	0.07 (93)	2.77	3.68	0.38 (86)	1.96 (47)
	in microcosms							
w/ groundwater & sediment materials								
live w/ lactate	0.42	2.07	0.05 (88)	0.45 (78)				
live w/ lactate & mixed culture	0.46	2.3	0.06 (87)	0.58 (75)				
w medium & sediment materials								
live w/ lactate	0.13	1.95	0.06 (54)	0.58 (70)				
live w/ lactate & mixed culture	0.14	2.6	0.04 (71)	0.42 (84)				

[a] 30-ml microcosms with C-58 DNAPL, established with groundwater and medium in April, 2004 (see Table 11). After 56 days of incubation, significant decreases in concentrations of C-56 and C-58 were observed in these microcosms (Table 12). These microcosms were amended with additional C-56 and C-58 after the initial 81 days of incubation, and served as biotic controls to examine differences from abiotic degradation of C-56 and C-58 in water and mineral medium.

[b] Incubated in a refrigerator at 4°.

[c] Data in parentheses represent percentage of removal of C-56 or C-58 after 10 or 16 days of incubation.

Report 6: Further Abiotic Investigations

- Performed by Qingzhong Wu, Ph.D.

OBJECTIVE

To further investigate abiotic mechanisms contributing to the fate of C-56 and C-58, a follow-up experiment to confirm the data shown in Table 15 was performed.

METHODS

Glass vials (20 ml nominal capacity) with 10 ml of anoxic, sterile water were autoclaved for 30 minutes at 121°C and equilibrated to room temperature before 15-20 µM (4.1-7.2 mg/l) C-56 and C-58were added. The vials were incubated at 4, 22, and 35°C in the dark.

An additional set of vials was autoclaved a second time following the addition of C-56 or C-58 to test the effect of high temperatures on the stability and fate of C-56 and C-58. For testing stability, C-56 or C-58 was added at two concentrations: a low concentration of 15-20 µM (4-6.9 mg/l) and a high concentration of 66-79 µM (18-27 mg/l). All vials were kept in the refrigerator after heat treatment. Chloride release was monitored in all vials using a Dionex DX-100 ion chromatograph equipped with an IonPac AS14A-4 x 250 mm column (Dionex, Co., Sunnyvale, CA). Sodium chloride was used to generate standards for chloride quantification. C-56 and C-58 were analyzed with GC/ECD following hexane extraction as described above.

RESULTS

Tables 16 and 17 show the C-56 and C-58 concentration changes and chloride release following incubation at 4, 22, and 35°C and after autoclaving (121°C for 30 minutes). In all vials, the decrease in C-56 and C-58 was concomitant with an increase in chloride concentrations, indicating an abiotic mechanism was involved in transformation of the target compounds. Each mole of C-56 and C-58 contains six and eight moles of chloride, respectively. Therefore, a small percentage of C-56 (4.9 – 13%) and C-58 (2.4 – 10.6%) were transformed via abiotic dechlorination under different temperatures and different initial concentrations of C-56 and C-58. About 0.3-0.4 and 0.08-0.14 moles of chloride were released per mole of C-56 and C-58 initially present, respectively, under abiotic conditions. Further, the data shown in Tables 16 and 17 indicate that temperature affects the abiotic dechlorination of C-56 and C-58.

Table 16. Change in the concentration of C-56 (μM) and chloride (μM) over time, in water amended with C-56. The data shown are averaged from duplicate vials.

	C-56 [μM]				Chloride [μM]			
	Initial conc.	After autoclave	7 days	14 days[b]	Initial conc.	After autoclave	7 days	14 days
Autoclave	15.0	3.6	-	2.8 (81)	6.1	17.5	-	17.8
Autoclave	65.5	32.6	-	24.2 (63)	17.7	46.1	-	45.1
4°C[a]	15.9	-	-	12.4 (22)	6.4	-	-	5.8
22°C	19.3	-	10.3	5.0 (74)	6.2	-	8.8	7.7
35°C	16.9	-	4.2	2.6 (85)	6.0	-	11.2	11.0

[a] stored in cold room at a constant temperature of 4°C.
[b] data in parentheses represent percentage of removal of C-56 or C-58 after 14 days of incubation.

Table 17. Change in concentration of C-58 (μM) and chloride (μM) in water amended with C-58 over the time. The data shown are averaged from duplicate vials.

	C-58 [μM]				Chloride [μM]			
	Initial conc.	After autoclave	7 days	14 days[b]	Initial conc.	After autoclave	7 days	14 days
Autoclave	19.9	5.8	-	5.5 (72)	1.6	17.1	-	18.4
Autoclave	78.8	16.0	-	14.6 (81)	8.1	51.6	-	59.5
4°C[a]	19.8	-	-	16.1 (19)	2.2	-		2.0
22°C	19.4	-	13.1	12.7 (35)	2.4	-	3.5	6.1
35°C	21.1	-	9.3	5.3 (75)	2.9	-	6.9	8.3

[a] stored in cold room at 4°C where the temperature was more constant than the refrigerator.
[b] data in parentheses represent percentage of removal of C-56 or C-58 after 14 days of incubation

GC/ECD analyses showed the formation of daughter products from C-56 and C-58 in vials that had been autoclaved. In an effort to identify the daughter products, mass selective analysis was performed on these samples following extraction hexane (2 ml) extraction of 2 ml aqueous samples with a Hewlett-Packard 6890 series GC equipped with an HP-5MS capillary column (30 m length, 0.25 mm inner diameter, 0.25 μm film thickness) and a Hewlett-Packard 5973 mass selective (MS) detector. GC operating conditions were identical to those described in the methods section of Report 3 for the GC/ECD analysis. One of the products formed had a mass of 204, and a library search identified 1,2,3,4-tetrachloro-1,3-cyclopentadiene ($C_5H_2Cl_4$, CAS #

695-77-2) as the closest mass spectrum match (86-90%). This compound was detected in autoclaved vials containing high concentrations of C-56 (18 mg/l) and C-58 (27 mg/l) but could not be detected in vials that were autoclaved or maintained at 22 and 35°C that received lower concentrations (<6.9 mg/l). Chloride release, however, was detected in these vials as well. The identification of this daughter product confirmed that an abiotic dechlorination pathway (or pathways) exists for C-56 and C-58. 1,2,3,4-tetrachloro-1,3-cyclopentadiene is not sold commercially, and hence, no authentic standard was available.

SUMMARY OF RESULTS AND CONCLUSIONS

Nucleic acid-based, microbial analysis of the original groundwater and sediment samples

1. DNA was obtained from all groundwater and sediment samples.
2. In all cases, bacterial universal primers yielded 16S rRNA gene amplicons, suggesting that PCR-amplifiable DNA suitable for nucleic acid-based analyses was obtained from all samples.
3. *Dehalococcoides* populations were detected using nested PCR, suggesting very low densities at all three sampling locations. This is an important observation because all known members of the *Dehalococcoides* group are obligate chlororespiring populations (i.e., require chlorinated compounds for growth).
4. Quantitative RTm PCR confirmed the presence of low, but quantifiable numbers of *Dehalococcoides* cells. This is not an altogether unexpected result, because *Dehalococcoides* cells are often present in low numbers at contaminated sites prior to enhanced bioremediation (e.g., biostimulation). Since *Dehalococcoides* populations are already present at the site, biostimulation may be considered as a possible alternative to bioaugmentation (adding a *Dehalococcoides*-containing inoculum), to increase the total *Dehalococcoides* population size. There was very little difference between the three sampling locations, in terms of *Dehalococcoides* abundance, suggesting that the populations subsist on some chlorinated substrate(s) at the site. C-56 and C-58 were detected also at the "clean" sampling location, indicating that chlorinated compounds are distributed throughout the site. The specific role of *Dehalococcoides* with respect to C-56 and C-58 dechlorination remains unknown. It remains to be determined if the parent compounds or daughter products formed in abiotic transformations are the substrates for *Dehalococcoides* species. .
5. 25-40% of the *Dehalococcoides* populations at the site possess a gene similar to the gene coding for the vinyl chloride reductive dehalogenase of *Dehalococcoides* sp. strain BAV1.
6. Other known groups of dechlorinators were not detected in the original sample materials prior to enrichment. Although the nested PCR approach is very sensitive, false negative results are possible. Hence, negative results must be interpreted carefully. Bacterial populations that occur below the detection limits of nested PCR prior to treatment can increase to detectable numbers following enhanced treatment (e.g., biostimulation).

Contaminant analysis of the original groundwater and sediment samples

1. Analysis of groundwater samples indicated that C-56 concentrations increased by roughly an order of magnitude between the "clean"(< 0.001 µg/ml), "mild" (0.01-0.03 µg/ml) and "DNAPL" (> 0.2 µg/ml) samples, while C-58 concentrations were low (< 0.001 µg/ml) in both the "clean" and "mild" groundwater samples and higher (0.003-0.0045 µg/ml) in the "DNAPL" sample. The analysis of replicate samples yielded similar values.
2. C-56 and C-58 were detected in the sediment collected at the "clean" location.
3. Concentrations of C-56 and C-58 in the "DNAPL" solid sample varied greatly among replicates suggesting the presence of free phase product.
4. In groundwater samples, higher concentrations of C-56 than C-58 were observed, whereas solid samples had higher concentrations of C-58 than C-56, suggesting that C-56 is more soluble than C-58.

Nucleic acid-based, microbial analysis of microcosms following enrichment

1. Community DNA was isolated from selected microcosm replicates (Table 6 and shaded in Table 13). Direct PCR detected *Dehalococcoides* in three microcosms, while nested PCR detected *Dehalococcoides* and *Dehalobacter* species in four microcosms. *Dehalobacter* species were not detected prior to enrichment but were easily detected following enrichment. Apparently, *Dehalobacter* species were present in the original samples in very low numbers, and were not detected with the nested PCR approach (i.e., false negative results were obtained). Interestingly, *Dehalobacter* 16S rRNA genes were readily detected following incubation, indicating that growth had occurred. Note that the known *Dehalobacter* populations are strict chlororespirers, only growing when a suitable chlorinated substrate is provided as the terminal electron acceptor. Hence, these findings implicate the involvement of *Dehalobacter* populations in reductive dechlorination processes occurring in the microcosms.
2. Quantitative RTm PCR data suggest that the *Dehalococcoides* community increased in size during the incubation period. This is a relevant finding indicating that *Dehalococcoides* populations were involved in reductive dechlorination reactions apparently occurring in the microcosms. The fraction of the *Dehalococcoides* community that carries the *bvcA* gene declined from 25% in the mild contaminated sediment to less than 5% in the microcosm, while the fraction carrying the *tceA* gene increased from non-detect to 5-20%. This findings strongly suggests that multiple *Dehalococcoides* populations are present at the site.

Fate of target contaminant in live microcosms and abiotic incubations

Microcosm Studies

1. The variability of C-56 and C-58 concentrations in the microcosms at the beginning of incubation suggests a high degree of variability within the cores received from EarthTech, and also suggests that mixing inside the glove box did not achieve a homogeneous preparation.
2. Significant reduction of C-56 and C-58 occurred in all microcosms, including in killed controls. In fact, concentrations of C-56 and C-58 in the autoclave-killed controls were close to the detection limit immediately after autoclaving. Also, immediately after autoclaving the concentrations of C-56 and C-58 in the killed controls were consistently less than the concentrations in the live microcosms after 206 days of incubation. This makes interpreting the microcosm data very complicated. As described in the section below, "Abiotic Investigations", abiotic mechanisms appear to be one of the causes for the decrease of C-56 and C-58 concentrations in all microcosms.
3. C-56 and C-58 transformations cannot be unambiguously attributed to biotic mechanisms due to the lack of a suitable abiotic control in the experiment described above. However, based on nucleic acid analyses, *Dehalococcoides* and *Dehalobacter* spp. increased in numbers during the 206-day incubation period. The known members of these genera are obligate chlororespiring organisms suggesting that these organisms may have used C-56, C-58 or their abiotic degradation/transformation products as electron acceptors. It is also possible that other chlorinated parent compounds such as hexachloroethane (for example) were present in the aquifer materials used to construct the microcosms, and that the obligate chlororespiring organisms used other chloroorganic compounds associated with the sediment material as electron acceptors.
4. All live microcosms produced methane, indicating that methanogenic activity and reduced conditions conducive for reductively dechlorinating bacteria had been established.
5. No C-56 and C-58 degradation products were detected in headspace samples (analyzed with GC/FID and GC/ECD) and in hexane extracts of water and soil (analyzed with GC/ECD) from microcosms.
6. PCE and *cis*-DCE were not degraded by native organisms, suggesting that (i) no, or very low numbers of chloroethene- dechlorinating bacteria are present at the site, or (ii) chloroethene-dechlorinating bacteria were inhibited.
7. *cis*-DCE accumulated in PCE-fed microcosms that were bioaugmented with a PCE-to-ethene-dechlorinating consortium. Complete dechlorination to ethene did not occur, suggesting that reductive dechlorination past *cis*-DCE was inhibited in the microcosms. The nature of this inhibition is unknown.

Abiotic Investigations

1. The abiotic transformation of C-56 and C-58 was observed in sterile mineral salts medium or water in the absence of solids.
2. Abiotic transformation of C-56 and C-58 in the sterile water containing no soil was supported by the identification of 1,2,3,4-tetrachloro-1,3-cyclopentadiene (TCCPD) by GC-MS. This degradation product could not be quantified because this chemical is not commercially available to generate a standard curve. Other degradation products were also observed in the sterile water containing no soil, but were not identified or quantified. No degradation products except chloride were observed in the other non-autoclaved vials that were incubated at various temperatures.
3. Abiotic transformation of C-56 and C-58 in the sterile water containing no soil was also supported by an increase in chloride concentrations in the aqueous phase at circumneutral pH values. The greatest increase in chloride ion concentrations occurred in the autoclaved vials, where the increase represents 10% (average of duplicates of C-56-amended vials) and 9% (average of duplicates of C-58-amended vials) of the total chlorine present in the C-56 and C-58 molecules at the start of the incubation period.
4. Preliminary findings from the abiotic investigations indicate that the rate of abiotic transformation of C-56 and C-58 depends both on temperature and on the presence or absence of light. The effects of temperature were observed in two separate investigations. Light appeared to be important in the one investigation in which it was tested.
5. Volatilization of C-56 or C-58 cannot explain loss from the live and killed microcosms. Only trace amount of C-56 and C-58 were detected in headspace samples using GC/ECD.

SUMMARY

Significant decrease of C-56 and C-58 concentrations occurred in all microcosms, including in killed controls. Evaporative losses of C-56 and C-58 from the vessels or sorptive losses to the Teflon-lined rubber septum cannot explain the decrease in concentrations. Incubations in the absence of microorganisms confirmed that abiotic pathways contribute to the fate of C-56 and C-58. The release of chloride and the absence of volatile or hexane-extractable products/intermediates suggest the formation of water-soluble products/intermediates. The intermediate 1,2,3,4-tetrachloro-1,3-cyclopentadiene may be water-insoluble, but not detected in the hexane extraction due to low concentrations and insufficient sensitivity of the GC/ECD analytical technique.

The nucleic acid-based analyses suggest that microbial reductive dechlorination occurred, and supported growth of *Dehalococcoccoides* and *Dehalobacter* populations. It is unclear if C-56 and C-58 abiotically formed degradation products, or other chlorinated compounds in the contaminated sites were the substrates for these chlororespiring populations (i.e., microbes that use chlorinated compounds as growth-supporting electron acceptors). Our investigations suggest that C-56 and C-58 were rapidly transformed at elevated temperatures. The product(s) of this abiotic transformation have not been identified. Further, our findings suggest that chlororespiring bacteria are involved in the transformation of C-56 and C-58 or the abiotically formed daughter product(s). The substrates and products of the microbial transformation process are unknown.

The effects of the PCE-to-ethene-dechlorinating consortium on the fate of C-56 and C-58 and their daughter products could not be evaluated. To delineate the contribution of microbial reductive dechlorination, the abiotic mechanisms, and the products of these pathways must be identified. Chloride release in live and killed microcosms could not be quantified and compared due to the chloride background.

The results of this study suggest that thermal treatment may efficiently reduce C-56 and C-58 concentrations and produce yet unidentified, chlorinated daughter products. The end products of combined abiotic/microbial degradation processes have not been identified, and it remains to be determined if these transformations result in detoxification.

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